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13. ABSTRACT ***(Maximum 200 Words)***

The *in vivo* relationship between epidermal growth factor (EGF) and prolactin/Jak/Stat signaling pathways in mammary gland development and tumorigenesis was explored in transgenic mice overexpressing the TGF alpha gene (TGFαTG) and lacking the prolactin-activated transcription factor, Stat5a (Stat5aKO/TGFαTG). We present evidence that Stat5a has a crucial role regulating programmed cell death (PCD) during mammary gland involution and the development of EGF-dependent tumorigenesis. TGFαTG mice exhibit delayed mammary gland involution and the absence of Stat5a facilitated involution-associated changes in morphology and the extent and timing of PCD. Stat5a remained phosphorylated through involution in TGFαTG suggesting that Stat5a is constituitively "activated" by the EGF signaling pathway. These results demonstrated TGFα epithelium is prevented from progressing into the normal pattern of involution and apoptosis by the "activated" form of Stat5a. This activated Stat5a affected EGF-receptor initiated mammary gland tumorigenesis. Overexpression of TGFα in mammary epithelium generates mammary hyperplasia and tumors. In the presence of an activated EGF receptor, deletion of Stat5a delayed initial hyperplasia and mammary tumor development by 6 weeks. These observations demonstrate that Stat5a is a survival factor, is required for mammary gland epithelium to resist regression and involution-mediated apoptosis and can influence the development of mammary gland tumorigenesis.

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FOREWORD

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Robin Humphrys 7.24.00.

PI - Signature Date

Table of Contents

Introduction

The objective of this research is to elucidate the relationship between the action of the transcription factor, Stat5a, and the regulation of programmed cell death in the mammary gland in order to establish the role of Stat5a in the development of breast cancer. This will be achieved through the development of a unique mouse model of breast cancer and the molecular analysis of the signaling pathways critical for the activation of this factor. This mouse model will permit evaluation of specific molecules involved in the Stat5a signaling pathway as modulators of involution and tumorigenesis.

Body

This research project began with interbreeding of the WAP-TGFα transgenic and Stat5a knockout mouse lines and to provide a detailed study of the effects of Stat5a deletion on WAP TGFα–induced tumor progression. This combination transgenic/knockout mouse line (TGFα/Stat5aKO) was established and was expanded through standard breeding protocols to generate sufficient experimental and control mice for the tumorigenesis studies. After 8 months of breeding and data collection it was evident that the absence of Stat5a did have an effect on the development of tumors in the WAP-TGFα transgenic tumor model. In the presence of TGFα overexpression, complete genetic deletion of Stat5a delayed initial mammary tumor development by 6 weeks. This preliminary data was presented in a poster format to the Breast Cancer Think Tank at Chantilly, Virginia. The alteration in the progression of involution was confirmed by histological analysis. WAP-TGFα transgenic mice have a significant delay in the onset of involution at the end of lactation. This delay is suggested to be one of the mechanisms for mammary tumor formation in this mouse model. It was observed that TGFα/Stat5aKO mammary glands underwent a significant reorganization and deletion of cell structure during involution. These changes replicated those observed during involution of a normal mammary gland. This observation suggested that the absence of Stat5a in the TGFα/Stat5aKO mice was allowing involution to occur and possibly reducing the tumorigenic capacity of the epithelium. Stat5a requires phosphorylation in order to be activated and is normally rapidly dephosphorylated at the beginning of involution(within 12 hours). Phosphorylation analyses of the tissues from WAP-TGFα transgenic mice revealed that Stat5a was inappropriately phosphorylated during involution. This provided evidence that the EGF signaling pathway was activating Stat5a during involution and preventing the regression of the epithelium. The EGF pathway was simultaneously activated implicating the EGF-dependent signaling kinase, MAPK, as the molecule initiating this phosphorylation event. This confirmed our earlier suspicions about the potential action of TGFα on Stat5a signaling in the mammary gland. Detailed molecular analysis of proliferation and programmed cell death levels revealed that there was an increase in apoptosis in the TGFα/Stat5aKO mouse when compared to the WAP-TGFα transgenic control gland. Suprisingly, apoptosis was increased during late pregnancy in the TGFα/Stat5aKO glands when normally cell death is low or absent in the wildtype gland. This was the first evidence that Stat5a can act as a survival factor for mammary epithelium. These experiments demonstrated that the absence of Stat5a affected the initiation of involution and the levels of apoptosis in the WAP-TGFα mammary gland. This data lead us to conclude that Stat5a can mediate signaling from the EGF receptor and can influence the progression of EGF-initiated breast cancer.

The absence of Stat5a delayed the initial appearance of tumor formation by 6-8 weeks. It did not completely abrogate the appearance of tumors and the rate of tumor formation was the same as the control group. This suggested that the process of involution can remove significant numbers of cells that could potentially contribute to the development of tumors. But it also suggested that there remained sufficient cells after the process of involution, that could respond to EGF-activated signaling, to initiate transformation of the epithelium. The data generated was sufficient to write and submit a manuscript "*Signal Transducer and Activator of Transcription 5a Influences Mammary Epithelial Cell Survival and Tumorigenesis*". The manuscript was accepted and published in Cell Growth and Differentiation in October, 1999.

The effect of Stat5a on the level and expression of cell death regulating molecules was examined through RNase protection assays. No significant change in the expression patterns of the Bcl-2 family members was observed. The activation of caspases 3, 6 and 9 molecules is currently under examination with protein and enzymatic assays. Initial experiments have been performed with specific kinase inhibitors to block the selected pathways that may phosphorylate and activate Stat5a protein. These experiments are aimed at determining the level and duration of exposure required for significant inhibition of Stat5a phosphorylation and possibly induction of Stat5a-dependent apoptosis. Implants were generated from plastic pellets combined with inhibitors for the MAPK, JAK and cAMP-dependent kinases. Results from these studies have demonstrated initial levels of kinase inhibitor required to effect minor morphological changes in the mammary epithelium immediately around the implant. No change in Stat5a status has been detected to date. This may require an increase in the dose and or duration of the kinase inhibitor. This data and the data published in the manuscript in October 1999, was presented in a platform talk at the Keystone Conference "*Advances in Breast and Prostate Cancer*" in Lake Tahoe in March of 2000.

A review article "*Transforming growth factor alpha and mouse models of human breast cancer*" was submitted and published in Oncogene in January 2000. This manuscript reviewed the known mouse models of human disease generated with the TGFα gene including my work with the TGFα/Stat5aKO.

This project is also interested in the possible mechanisms of compensation or competition for Stat5a action by related family members. Based on conclusions from our work with the TGFα/Stat5aKO mouse model a collaboration was setup with Dr. David Levys lab at New York Medical Center, New York to study the role of Stat3 in the development and transformation of the mammary gland. I have developed another combination transgenic-knockout mouse model to examine the role of this molecule in mouse mammary cancer and development. This mouse model is currently being developed.

Key Research Accomplishments

1. **Paper:** *Signal Transducer and Activator of Transcription 5a Influences Mammary Epithelial Cell Survival and Tumorigenesis* published in Cell Growth and Differentiation 10:685-694, October, 1999.
2. **Paper:** *Transforming growth factor alpha and mouse models of human breast cancer* published in Oncogene (review article) 19: 1085-1091, January ,2000.
3. **Platform talk** :Presented at Keystone Meeting "Advances in Breast and Prostate Cancer" , March , 2000.
4. **Poster:** Presented at Breast Cancer Think Tank Meeting, July, 1999. Received poster award to attend meeting.
5. Generated the combined WAP-TGFα transgenic Stat5a knockout mouse model of breast cancer.
6. Demonstrated Stat5a has a cell survival function in the mammary epithelium.
7. Demonstrated a connection between Stat5a activity and the development of mammary cancer.
8. Established that Stat5a has a regulatory role in the involution of the mammary gland .
9. Used an *in vivo* model of breast cancer to demonstrated that the regulation of the activity a transcription factor can affect the development and progression of transformation in the mammary gland.
10. Applied for several faculty positions based on research from this work. University of Washington, University of Arizona , University of Oregon, University of Washington (St. Louis).
11. Collected tissue from all animal models to generate a tissue bank of various stages of WAP-TGFα transgenic and TGFα/Stat5aKO mammary glands. These tissues were used for various mammary gland analyses within and outside our lab. Including microarray analysis of gene expression of tumor types in the mammary gland. The data garnered from this analysis was used to prepare a manuscript from the Hennighausen lab on the development of a microarray analysis program(submitted).
12. Developed a collaboration to study the role of a related Stat molecule in the same breast cancer mouse model.

Reportable Outcomes

1. **Paper:** *Signal Transducer and Activator of Transcription 5a Influences Mammary Epithelial Cell Survival and Tumorigenesis* published in Cell Growth and Differentiation 10:685-694, October, 1999.
2. **Paper:** *Transforming growth factor alpha and mouse models of human breast cancer* published in Oncogene (review article) 19: 1085-1091, January ,2000.
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5. Generated the combined WAP-TGFα transgenic/Stat5a knockout mouse model of breast cancer.

Signal Transducer and Activator of Transcription 5a Influences Mammary Epithelial Cell Survival and Tumorigenesis[1]

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Abstract

The mammary gland undergoes extensive tissue remodeling and cell death at the end of lactation in a process known as involution. We present evidence that the prolactin-activated transcription factor signal transducer and activator of transcription 5a (Stat5a) has a crucial role in the regulation of cell death during mammary gland involution. In a transforming growth factor-α transgenic mouse model that exhibited delayed mammary gland involution, the absence of Stat5a facilitated involution-associated changes in morphology of the gland and the extent and timing of programmed cell death. These Stat5a-dependent changes also affected epidermal growth factor receptor-initiated mammary gland tumorigenesis. Overexpression of the transforming growth factor α transgene in the mammary epithelium reproducibly generated mammary hyperplasia and tumors. In the presence of the activated epidermal growth factor receptor, deletion of Stat5a delayed initial hyperplasia and mammary tumor development by 6 weeks. These observations demonstrate that Stat5a is a survival factor, and its presence is required for the epithelium of the mammary gland to resist regression and involution-mediated apoptosis. We also suggest that Stat5a is one of the antecedent, locally acting molecules that initiate the process of epithelial regression and reorganization during involution.

Introduction

Involution is the phase of mammary gland development that remodels the molecular and morphological characteristics of the gland after lactation. The systemic levels of prolactin, one of the dominant hormones in the stimulation and maintenance of lactational competence of the gland, decreases at the onset of involution (1). The characteristics of the biological activity and regulation of prolactin argue for prominent involvement of this systemic hormone in the induction of mammary gland involution. Prolactin secretion is tightly regulated and is stimulated by suckling. The half-life of prolactin is very short in the blood, and there is evidence that exogenous treatment with prolactin can delay involution at certain stages of development (2, 3). Despite the clear role of this systemic hormone in the initiation of involution, the role of its subservient signal transduction pathways in the regulation of the morphological and transcriptional changes of involution is unknown. The prolactin-activated transcription factor Stat5a[3] is essential for the development and differentiation of the mammary gland (4). Stat5a phosphorylation sharply declines within 12 h after weaning, suggesting a role in the onset of involution. In fact, Stat5a has been implicated in the regulation of apoptotic processes in other tissues (5, 6).

Mammary gland involution can be separated into two molecularly and morphologically distinct phases. The first phase is defined by changes in the expression patterns of genes involved in milk synthesis (WAP and ornithine decarboxylase), cell survival and death (Bax, Bcl-2, Bcl-x_s, SGP-2, interleukin-converting enzyme 1, and p53), and proteins that act to regulate potential survival factors (insulin-like growth factor-1, insulin-like growth factor-binding protein 5, and TGF-β) and modulate interactions with the extracellular matrix (tissue inhibitor of metalloproteinase 1; Refs. 3 and 7–11). Importantly, dephosphorylation of the prolactin-dependent transcription factors Stat5a and Stat5b can be detected within 12 h of the initiation of involution (12). Despite the high levels of apoptosis early in involution, the integrity of the alveolar basement membrane is not disrupted. The second phase of involution is characterized by increases in the expression of proteins and genes involved in reorganizing the extracellular matrix (gelatinase 1, stromelysin 1, and urokinase plasminogen activator) and abrogation of milk gene expression. These molecular changes precede the dramatic degradation of the lobuloalveolar acini and the absorption of apoptotic cells. It is presumed that changes in the concentrations of the systemic hormones prolactin, oxytocin, and progesterone, in concert with changes in the activity of locally acting factors, initiate and stimulate the process of involution.

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[3] The abbreviations used are: Stat, signal transducer and activator of transcription; TGF, transforming growth factor; Jak, janus kinase; MAPK, mitogen-activated protein kinase; WAP, whey acidic protein; EGFR, epidermal growth factor receptor; BrdUrd, bromodeoxy uridine; WT, wild-type; PCD, programmed cell death; EGF, epidermal growth factor; TBST, Tris buffered saline Tween; TUNEL, terminal deoxynucleotidyl transferase-mediated uridine nick end labeling; ERK, extracellular signal-regulated kinase.

The importance of the Stat proteins in the growth and differentiation of many cell types is exemplified by their ubiquitous expression and widespread utilization in the signal transduction pathways of multiple cytokines (13). Stats are capable of transmitting both differentiative and proliferative signals, depending on the cellular and receptor context (14, 15). In the mammary gland, Stat5a activation by prolactin is required for functional differentiation of the epithelium during pregnancy and lactation (4, 16). Singular tyrosine phosphorylation by the prolactin receptor-associated intracellular kinase Jak-2 is obligatory to maintain Stat5a transcriptional activity (17). However, novel alternative mechanisms of regulating Stat5a activity have been identified. Prolactin-activated signal transduction can tyrosine- and serine-phosphorylate multiple isoforms of the Stat5a molecules (18). Furthermore, truncated forms of Stat5a have transcriptional dominant negative activity (19, 20) and have been linked to the regulation of apoptosis (5). In the liver, EGF can stimulate phosphorylation and nuclear translocation of the Stat5a isoform, Stat5b (21). Importantly, serine phosphorylation of Stat5a by the prolactin-independent, MAPK pathway has been identified, (22) including a direct association between Stat5a and the serine kinase ERK-1/2(MAPK) (23). Serine phosphorylation by the MAPK pathway is required for transcriptional activity of Stat1 and Stat3, but serine phosphorylation is not obligatory for prolactin-dependent transcriptional activity of Stat5 proteins (24). Taken together, these data imply that Stat5a activity can be regulated by the prolactin and EGF signaling pathways and possesses diverse functional roles within a single cell that are dependent on its phosphorylation state and expressed isoforms.

Initiation and progression of breast cancer rely on the inappropriate temporal and qualitative activity of normal cellular signaling pathways (25). These alterations in signaling activity often disrupt the normal mechanisms of mammary gland development. In one mouse model (WAP-TGF-α), overexpression of TGF-α, which binds to and activates the EGFR, delays mammary involution and transforms the mammary epithelium (26). The prolactin signaling pathway has been implicated in loss of normal epithelial differentiation (4, 16) and transformation (27, 28). The role of Stat5a in tumorigenesis is implicated by the appearance of Stat5a overexpression in leukemic (29, 30) and oncogenic (31) transformed cells. Additionally, constitutive activation of the Jak/Stat pathway occurs in multiple leukemia-, v-abl-, and human T-cell lymphotrophic virus-transformed T cells (32, 33), and specific inhibition of constitutive Jak-2 activity in acute lymphoblastic leukemia can block cell growth and induce cell death (34). We used the WAP-TGF-α transgenic and Stat5a-null mouse models to explore the *in vivo* role of Stat5a in mammary gland involution and tumorigenesis.

Results

Delayed Mammary Involution in the TGFαTG Mice Is Curtailed in the Absence of Stat5a. The contribution of Stat5a in regulating involution and the establishment of mammary tumors was investigated in mice that carried the TGF-α transgene (TGFαTG mice) or were null for the *Stat5a* gene (Stat5aKO mice) and in *Stat5a*-null mice that expressed the transgene (Stat5aKOTGFα mice). H&E-stained mammary glands from TGFαTG, Stat5aKO, Stat5aKOTGFα, and WT nontransgenic control mice at day 18 of pregnancy and days 1, 3, and 7 of involution were examined by light microscopy (Fig. 1). Morphological differences were observed between the TGFαTG and Stat5aKOTGFα mammary glands within the first pregnancy and involution. TGFαTG mammary glands contained hyperproliferative alveoli and ducts. Alveolar lumina contained heterogenous eosin-positive staining material. A significant increase in the number of stromal cells was evident, in addition to increased deposition of stromal collagen (Fig. 1*l*). Involution was delayed in the TGFαTG mice, as reported previously (26). Some cell loss and condensation of lobuloalveolar structures was observed during involution, but the gland lacked the dramatic epithelial regression of the WT gland (Fig. 1, *n versus p*). Mammary tissue from pregnant Stat5aKOTGFα mice was histologically similar to pregnant tissue from TGFαTG mice but contained more epithelium than observed in the glands of Stat5aKO mice (Fig. 1, *a–d*). Secretory structures appeared to be more uniform and lacked the observed heterogeneous precipitations in the alveolar lumens. During involution, the Stat5aKOTGFα gland remodeled more rapidly and extensively than the TGFαTG gland (Fig. 1, *o versus p*). This effect was enhanced after subsequent rounds of pregnancy and involution (data not shown). The primary and secondary ducts of the Stat5aKOTGFα mice lacked concentrations of hyperplastic cells like those found in the TGFαTG mice. On rare occasions, hyperplastic regions were evident after three or more rounds of pregnancy in Stat5aKOTGFα mice (data not shown).

Apoptosis and Proliferation Levels Are Altered in the Stat5aKOTGFα and TGFαTG Mice. High levels of apoptosis, decreased proliferation, and the collapse of lobuloalveolar structures in the mammary epithelium are hallmarks of involution in the mammary gland (7). To establish and define the characteristics of mammary involution in the Stat5aKOTGFα and TGFαTG mice, the levels of apoptosis and proliferation were analyzed by TUNEL and BrdUrd immunohistochemistry, respectively (Tables 1 and 2). WT glands exhibited increased levels of apoptosis within 1 day of the initiation of weaning and exhibited maximal levels at day 3, as expected (9, 35). Consistent with an inhibition or delay in involution in the TGFαTG gland, the number of apoptotic cells was slightly reduced at day 1 of involution compared with WT and Stat5aKOTGFα glands (Fig. 2*A*). Apoptosis was reduced in the TGFαTG gland at day 7 when compared with WT gland at day 7. Whereas Stat5aKOTGFα mammary glands at day 18 of pregnancy ($P = 0.02$) and at day 1 of involution ($P = 0.015$) had significantly higher levels of apoptosis than TGFαTG and WT mammary glands over the same time period, the levels were similar at days 3 and 7. Interestingly, Stat5aKO mammary glands also displayed significantly high levels of apoptosis at day 18 of pregnancy and day 1 of involution ($P = 0.03$).

Levels of proliferation, as measured by BrdUrd incorporation, were equivalent in mammary tissue of pregnant Stat5aKOTGFα, TGFαTG, Stat5aKO, and WT mice (Fig. 2*B*). Generally, 6–10% of the cells in the pregnant gland were

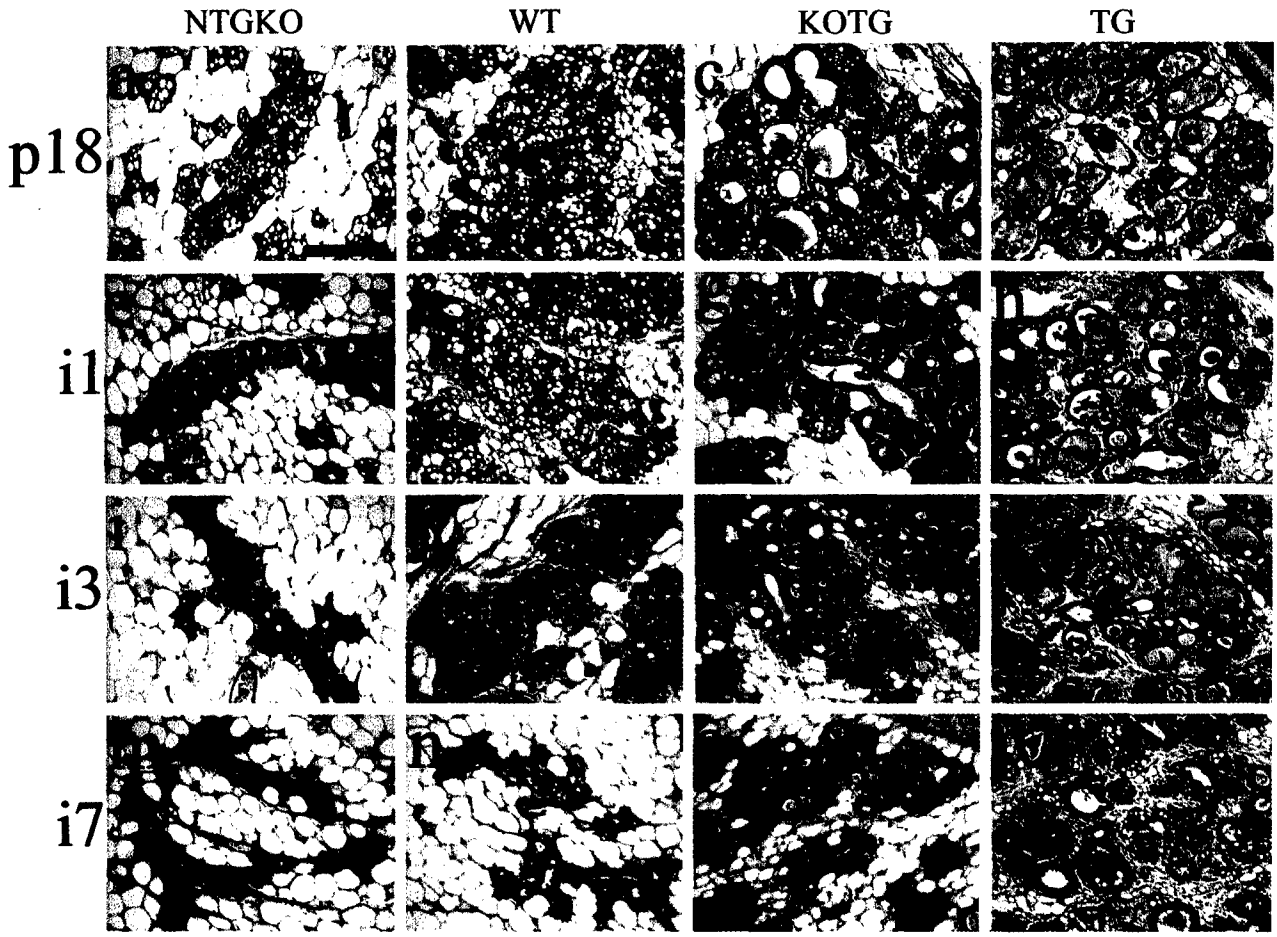

Fig. 1. Involution is enhanced in the absence of *Stat5a*. H&E staining of inguinal mammary glands from Stat5a-null nontransgenic (*NTGKO*), WT, Stat5a-null TGF-α transgenic (*KOTG*), and TGF-α transgenic (*TG*) mice at 18 days of pregnancy (*p18*, *a–d*) and days 1 (*i1*, *e–h*), 3 (*i3*, *i–l*), and 7 (*i7*, *m–p*) of involution. Samples were collected from mice during or immediately after their first pregnancy. Compare epithelial condensation at day 3 and day 7 of involution in the WT (*j*) *versus* the KOTG (*k*) and TG (*l*). *Bar* in *a*, 200 μm.

Table 1 Percentage of apoptotic cells detected with TUNEL

Values are the mean value ± SE. Superscript symbols denote mean values that were significantly different from the WT mean value of the same day.

	KOTG	TG	WT	NTGKO
Preg. day 18	4.21 ± 0.65[a]	0.86 ± 0.31	1.47 ± 0.56	2.61 ± 0.52
Involution day 1	9.01 ± 1.46[b]	2.01 ± 0.49	3.21 ± 0.96	7.65 ± 1.19[c]
Involution day 3	6.96 ± 0.41	4.92 ± 1.92	5.97 ± 0.75	4.35 ± 0.14
Involution day 7	3.32 ± 0.74	1.75 ± 0.56	4.56 ± 1.42	4.16 ± 1.89

[a] $P = 0.02$.
[b] $P = 0.015$.
[c] $P = 0.03$.

Table 2 Percentage of proliferating cells detected with BrdUrd labeling

Values are the mean ± SE. Superscript symbols denote mean values that were significantly different from the WT mean value of the same day.

	KOTG	TG	WT	NTGKO
Preg. day 18	10.30 ± 5.16	10.03 ± 1.77	6.97 ± 1.18	4.09 ± 1.72
Involution day 1	7.40 ± 2.12[a]	3.63 ± 1.33	1.38 ± 0.49	1.53 ± 0.23
Involution day 3	6.43 ± 0.64[b]	4.00 ± 1.84	0.67 ± 0.27	0.96 ± 0.15
Involution day 7	6.87 ± 0.80[c]	5.62 ± 1.05[d]	0.07 ± 0.07	0.65 ± 0.28

[a] $P = 0.05$.
[b] $P = 0.001$.
[c] $P = 0.001$.
[d] $P = 0.007$.

labeled with BrdUrd (Fig. 2*B*). Proliferation was low in involuting tissue from Stat5aKO and WT mice (<1%). In contrast, the TGFαTG and Stat5aKOTGFα glands had detectable, persistent proliferation levels of 4–7% throughout involution. Apoptotic cells were observed in Stat5aKOTGFα mammary glands at day 18 of pregnancy and day 1 of involution in animals labeled exclusively with BrdUrd (Fig. 2*C*, *arrows*). The appearance of apoptotic, BrdUrd-labeled cells demonstrated that DNA synthesis in these cells preceded the initiation of PCD.

WAP-TGF-α Transgene Expression Is Present throughout Mammary Gland Development in Stat5aKOTGFα and TGFαTG Mice. The WAP gene promoter has been exploited to target high-level expression of transgenes, including TGF-α, to the mammary epithelium of pregnant mice (36–40). To assure that the hormonal and morphological changes

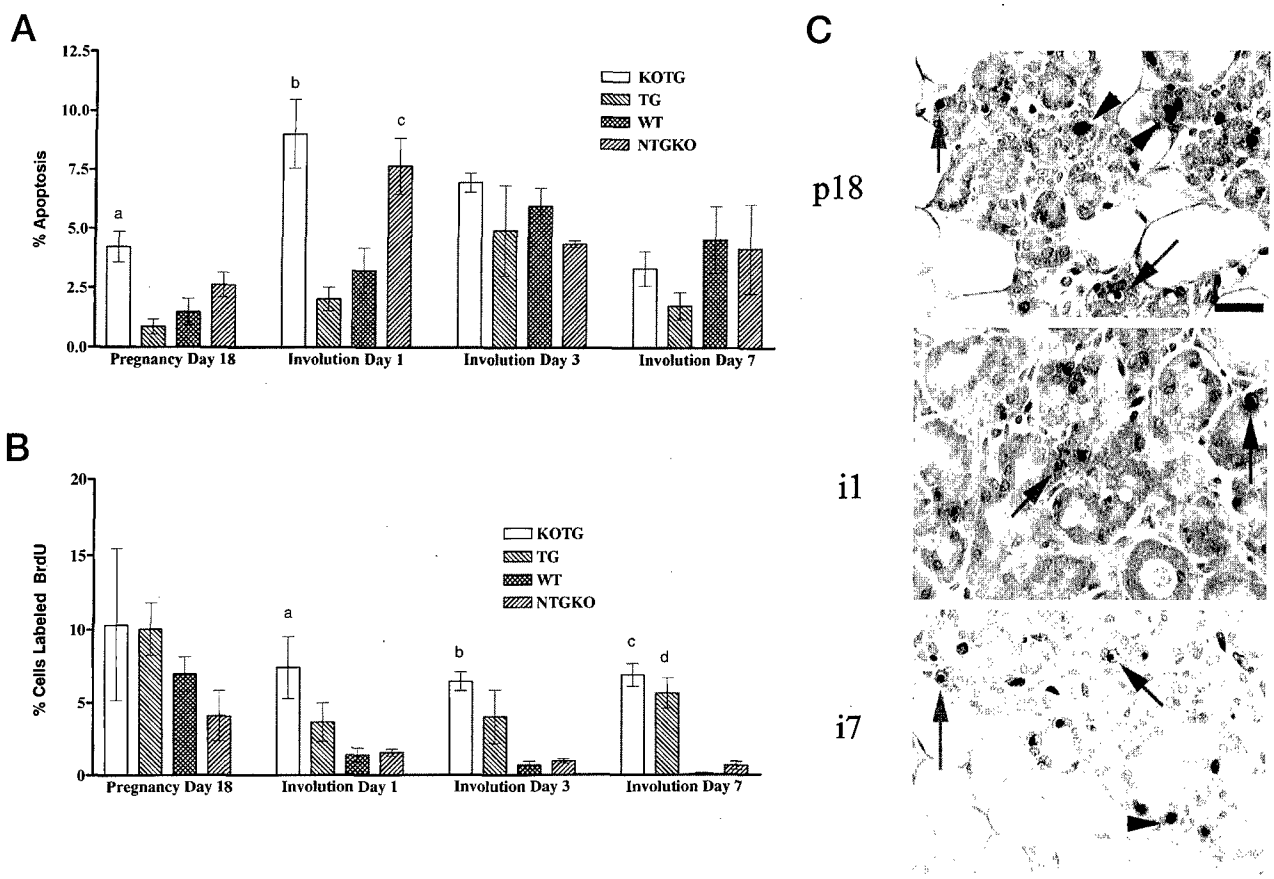

Fig. 2. *A*, KOTG mammary glands display elevated levels of apoptotic cells during pregnancy and early involution. Apoptosis levels in Stat5a-null TGF-α transgenic (*KOTG*), TGF-α transgenic (*TG*), Stat5a-null nontransgenic (*NTGKO*), and WT mammary glands after analysis by the TUNEL technique. Values represent the percentage of cells (means $\pm$ SE) displaying the label in a minimum population of 1000 cells. Analysis was performed on mice during or immediately after their first pregnancy. Serial sections of tissue from the same mouse were used for the determination of proliferation and apoptosis. A minimum of three mice were analyzed from each genotype (see "Materials and Methods."). Total number of cells counted, 7.5×10^5. *a*, *b*, and *c* denote significant differences between the KOTG and NTGKO values when compared with apoptosis levels in WT glands at day 18 of pregnancy and day 1 of involution. Specific values for Fig. 2, *A* and *B*, are shown in Tables 1 and 2. *B*, proliferation is higher in TG and KOTG mammary glands through involution. Proliferation levels in Stat5a-null TGF-α transgenic (*KOTG*), TGF-α transgenic (*TG*), Stat5a-null nontransgenic (*NTGKO*), and WT mammary glands after analysis by BrdUrd labeling. Values represent the percentage of cells (means $\pm$ SE) displaying the label in a minimum population of 1000 cells. Analysis was performed on mice during or immediately after their first pregnancy. Serial sections of tissue from the same mouse were used for determination of proliferation and apoptosis. A minimum of three mice were analyzed from each genotype. Total number of cells counted, 6.5×10^5. *a*, *b*, *c*, and *d* significant differences between TG and KOTG samples when compared with WT at day 1, 3, and 7 of involution. *C*, proliferation analysis of mammary gland in Stat5a-null TGF-α transgenic mammary glands. This figure demonstrates the appearance of BrdUrd-labeled apoptotic cells in late pregnancy (*p18*) and days 1 and 7 of involution (*i1* and *i7*). Mice were injected 2 h before sacrifice with 20 μg/g body weight BrdUrd, as described in "Materials and Methods." *Gray arrows*, BrdUrd-labeled apoptotic cells. *Black arrowheads*, intact BrdUrd-labeled nuclei. *Bar* in *p18*, 100 μm.

associated with involution and the absence of Stat5a did not disrupt transgene expression, TGF-α expression was evaluated in mammary tissue of pregnant and involuting TGFαTG, WT, and Stat5aKOTGFα mice. Northern blot analysis of TGF-α transgene expression in TGFαTG and Stat5aKOTGFα mice revealed expression of an appropriately sized transcript at day 18 of pregnancy. Transgene expression persisted through days 1, 3, and 7 of involution in multiple animals (Fig. 3). This expression pattern deviates from the expression of the endogenous WAP gene, which declines at the initiation of involution (41). Endogenous TGF-α expression was only detected in an overloaded lane containing wildtype tissue at day 1 of involution. TGF-α transgene expression persisted through multiple rounds of pregnancy (three to nine rounds) in TGFαTG and Stat5aKOTGFα mice (data not shown).

The EGFR-activated Serine Threonine Kinase MAPK Remains Active throughout Involution. Binding of TGF-α to the EGFR initiates receptor dimerization and inherent kinase activation, which stimulates the Ras/Raf/Mek signaling cascade. To establish that the EGFR-dependent pathway was being activated by the expression of the TGF-α transgene, Western blot analysis was performed on protein extracts from Stat5aKOTGFα, WT, Stat5aKO, and TGFαTG animals with an antibody that only recognizes the active (phosphorylated) form of MAPK. Active MAPK was detected in TGFαTG and Stat5aKOTGFα mammary glands at day 18 of pregnancy and days 1, 3, and 7 of involution (Fig. 4, *A* and *B*). Increased levels of the active form of this enzyme were detectable after multiple rounds of pregnancy in the TGFαTG and Stat5aKOTGFα mammary glands. Very low levels of

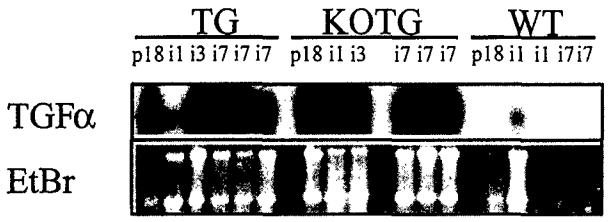

Fig. 3. TGF-α transgene expression persists through involution in KOTG and TG mammary glands. Northern analysis of TGF-α expression in *TG*, *KOTG*, and *WT* mammary glands. Total RNA (20 μg) was separated and analyzed on a single Northern blot. Each lane represents RNA from a single animal. Samples were collected from mice during or immediately after their first pregnancy. The TGF-α blot was exposed for 4 h at −70°C. *EtBr*, ethidium bromide staining of RNA.

active MAPK were detected in the nontransgenic Stat5aKO gland (Fig. 4*C*). Levels were also very low in pregnant and involuting WT mammary glands (Fig. 4*D*). A significant level of MAPK is present in the lactating, WT mammary gland.

Stat5a Proteins Are Phosphorylated during Involution in TGFαTG and Stat5aKOTGFα Mice. Stat5a is phosphorylated on tyrosine 694 by Jak-2, which is associated with the dimerized prolactin receptor (13). Stat5a must remain phosphorylated to maintain its transcriptional activity. One of the earliest molecular events after the onset of involution is the dephosphorylation of Stat5a and Stat5b (42). Immunoprecipitation and Western blot analysis of protein extracts from TGFαTG mice detected high levels of tyrosine-phosphorylated Stat5a and Stat5b at days 1, 3, and 7 of involution (Fig. 5). Conversely, the phosphorylation of Stat5a and Stat5b was decreased at day 1 and remained low to day 7 of involution in WT controls, as reported previously and shown in Fig. 5*B* (42). Additionally, tyrosine-phosphorylated Stat5a and Stat5b were also detected by immunoprecipitation and Western blot analysis in multiple individual TGFαTG tumors (data not shown). Stat5aKOTGFα mammary glands, lacking any Stat5a protein, were assayed for the phosphorylation of Stat5b to determine whether there was a compensation for the absence of Stat5a phosphorylation. A slight increase in Stat5b phosphorylation was detected on days 1, 3, and 7 of involution relative to WT controls.

TGF-α-initiated Mammary Tumorigenesis Is Delayed in Stat5aKOTGFα Mice. The rat TGF-α transgene under control of the WAP gene promoter induces mammary tumors in mice bred *ad lib* after three pregnancies (26). The contribution of Stat5a to the establishment of mammary hyperplasias and tumors was investigated in TGFαTG and Stat5aKOTGFα mice. Stat5aKOTGFα and TGFαTG mice were bred *ad lib* and examined after each round of pregnancy for palpable hyperplasias and tumors. All 48 TGFαTG mice contained palpable hyperplasia in multiple glands by the third pregnancy. TGFαTG mammary glands consistently displayed a swollen appearance by the second pregnancy, emblematic of hypertrophic glands and hyperplasias (data not shown). Gross histological examination revealed the presence of multiple hyperplastic alveolar nodules in multiple glands. TGFαTG glands also contained fluid-filled cysts and stromal proliferation. Some removed tumors were characterized by

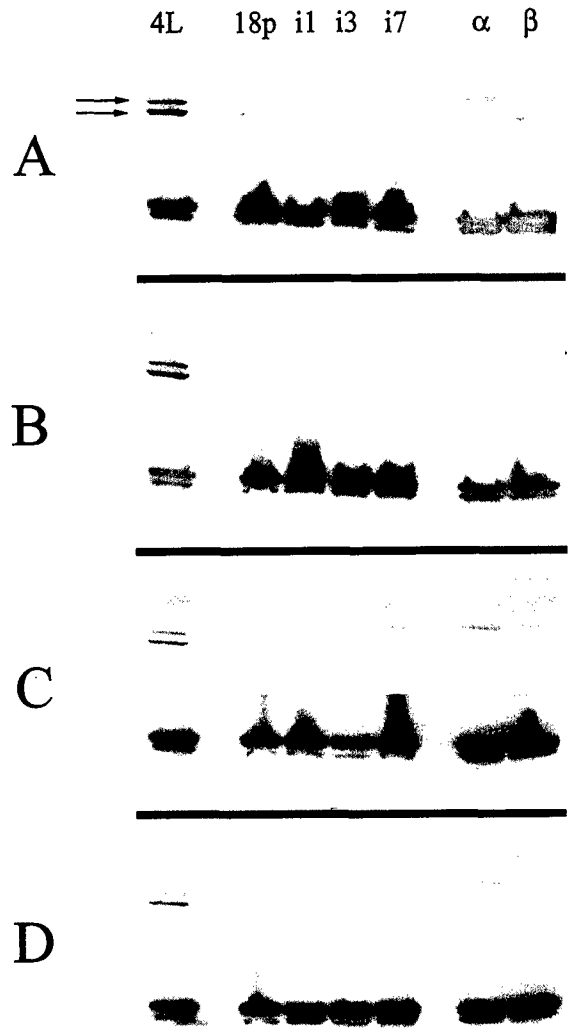

Fig. 4. MAPK is active during pregnancy and involution in TG and KOTG mammary glands. Phosphorylation of MAPK was examined by Western blot analysis with an antibody specific for the active phosphorylated form of MAPK and is shown in the *top panel* of each section. *18p*, *i1*, *i3*, and *i7* represent samples collected from TG (*A*) KOTG (*B*), NTGKO (*C*), and WT (*D*) mammary glands. *4L*, a control sample from a WT, 4 day lactating mammary gland. α, a sample from a KOTG mammary gland after three pregnancies. β, a TG mammary gland after three pregnancies. Each immunoblot was stripped and reblotted with an antibody to MAPK to determine loading, and the result is shown directly under the phosphorylated MAPK immunoblot in each panel. *Arrows* in *A* indicate the two subunits of MAPK, ERK-1 (*top*) and ERK-2 (*bottom*).

abnormal hyperproliferation of glandular structures with relatively little hyperplasia. The latency of mammary hyperplasia and tumor appearance and the histology of the hyperplasias and tumors were consistent with the results described previously (26). Hyperplasia and hypertrophy appeared less frequently in the Stat5aKOTGFα mammary gland. There was a significant difference in the rate of initial hyperplasia and tumor formation between the TGFαTG and Stat5aKOTGFα mice, ($P = 0.003$; Fig. 6). The initial appearance in the Stat5aKOTGFα mice was delayed 1.5 months compared with the TGFαTG mice. In Stat5aKOTGFα mice

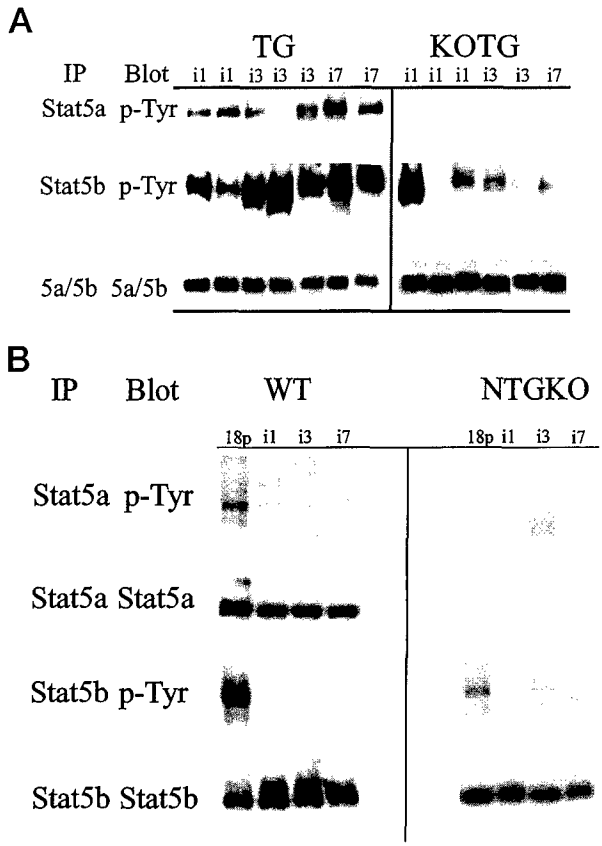

Fig. 5. *A*, Stat5a remains phosphorylated during involution in TG mammary glands. Stat5a and Stat5b phosphorylation was examined in *KOTG* and *TG* mice mammary glands. Protein extracts were immunoprecipitated with anti-Stat5a antibodies (*Stat5a*, 1:2 × 10^5) or anti-Stat5b antibodies (*Stat5b*, 1:1 × 10^5) overnight. Immunoprecipitates were separated by PAGE and immunoblotted with anti-phosphotyrosine (*p-Tyr*, 1:1 × 10^3) antibodies. Samples were collected from mice during or immediately after their first pregnancy. Immunoblots probed initially with p-Tyr were stripped and reblotted with anti-Stat5a antibodies in the TG samples (*5a/5b*; *5a/5b*) or anti-Stat5b antibodies in the KOTG samples (*5a/5b; 5a/5b*), respectively, to determine protein loading. *i1, i3*, and *i7*, days 1, 3, and 7 of involution, respectively. *B*, Stat5a and Stat5b are dephosphorylated during involution. Stat5a and Stat5b phosphorylation was examined in *NTGKO* and *WT* mice mammary glands. Protein extracts were immunoprecipitated with anti-Stat5a antibodies (*Stat5a*, 1:2 × 10^5) or anti-Stat5b antibodies (*Stat5b*, 1:1 × 10^5) overnight. Immunoprecipitates were separated by PAGE and immunoblotted with anti-phosphotyrosine (*p-Tyr*, 1:1 × 10^3) antibodies. Samples were collected from mice during or immediately after their first pregnancy. Immunoblots probed initially with p-Tyr were stripped and reblotted with anti-Stat5a antibodies or anti-Stat5b antibodies to determine protein loading. *p18, i1, i3*, and *i7*, day 18 of pregnancy and days 1, 3, and 7 of involution, respectively.

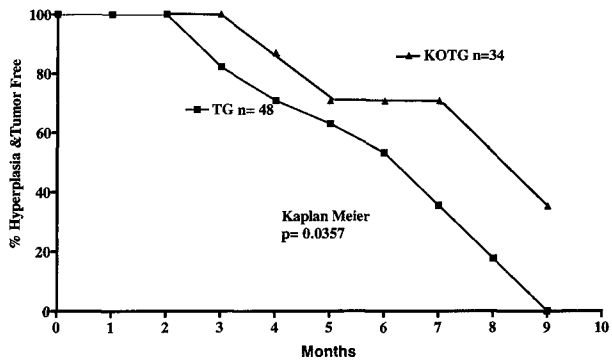

Fig. 6. Absence of Stat5a delays EGFR-mediated hyperplasia and tumor formation. Kaplan-Meier plot of the percentage of hyperplasia and tumor-free *KOTG* and *TG* mice over a 9-month time period. All mice were examined at each pregnancy for palpable tumors. There is a significant difference between the two curves (P = 0.0357). TG, n = 48. KOTG, n = 34.

that did develop hyperplasias or tumors, usually only a single gland was involved, as compared with multiple gland involvement in the TGFαTG mice.

Discussion

Loss of Stat5a Activity Is an Early Event in Mouse Mammary Gland Involution. Dephosphorylation and hence inactivation of the transcription factors Stat5a and Stat5b and *de novo* phosphorylation of Stat3 occur in the first phase within several hours of pup removal (42), implicating the Stats and prolactin as early regulatory molecules in the regression of the gland. Using a combination of transgenic and gene deletion mouse models, we have now demonstrated a role for Stat5a in involution that is dependent on its phosphorylation and can be regulated by constitutive EGFR signal transduction. Involution is abrogated on continued activation of the EGFR (and Stat5a) through the expression of a TGF-α transgene. In mammary tissue of TGFαTG mice, Stat5a remained phosphorylated, as did Stat5b. However, on deletion of the Stat5a gene, involution proceeded unabated, despite expression of the TGF-α transgene. Rapid involution in the absence of Stat5a was accompanied by increased levels of apoptotic cells. Our experiments not only demonstrate that Stat5a is a survival factor *in vivo* but also show that its presence is mandatory to avoid the initiation of PCD in mammary epithelial cells.

Despite the lack of known genetic targets for Stat5a in the involuting gland, there is evidence to suggest that prolactin and Stat5a can control cell survival. Activation of Stat5a has been implicated in the inhibition of apoptosis in T cells through interleukin 2 (6) and interleukin 9 (43) signaling. In addition, selection of apoptosis-resistant immature T cells leads to the activation of a dominant negative form of Stat5a (5). Prolactin can also stimulate cell survival in mammary epithelial cells *in vitro* (44), and prolactin and growth hormone can promote cell survival in the involuting rat mammary gland (2, 3). The presence of apoptotic cells in the mammary tissue of pregnant mice that lack the *Stat5a* gene and express the TGF-α transgene suggests that cell dependence on the presence of phosphorylated Stat5a precedes the actual systemic hormonal switch into involution. This observation also implies that the Stat5a-dependent mechanism of apoptosis induction may be hormonally independent. We did not observe this apoptotic phenomena in the WT and TGF-α transgenic mice. This increased number of apoptotic cells during pregnancy was also detected in BrdUrd-labeled Stat5aKOTGFα mice. Analysis of these glands revealed the presence of BrdUrd-labeled apoptotic cells at day 18 of pregnancy and at days 1, 3, and 7 of involution. These cells replicated their DNA and then proceeded directly into PCD.

This association between the cell's ability to replicate its DNA and the progression into the apoptotic program has been reported previously (45). Cells may be programmed to initiate PCD before they enter the cell cycle; consequently, BrdUrd-labeled cells undergoing apoptosis are detected. Interestingly, not all the cells in the Stat5aKOTGFα pregnant gland undergo PCD. This suggests that some cells have obviated the requirement for Stat5a, possibly through an unknown compensatory mechanism. Alternatively, these cells may have a unique differentiation state that precludes them from entering the apoptotic pathway.

Despite persistent phosphorylation of Stat5a in involuting mammary tissue, TGFαTG mice did undergo involution-dependent epithelial reorganization and activation of PCD. This morphological change was characterized by moderate condensation of alveolar structures and loss of cells through the induction of PCD. We propose that the dephosphorylation of Stat5a is not the only guiding factor for apoptosis progression in the involuting mammary gland and that other parallel signals may be required for the complete induction of both phases of involution (12).

Transgenic TGF-α Activates the EGFR and Ensures Persistent Phosphorylation of Stat5a. TGF-α stimulates the EGFR and activates MAPK through the Ras/Raf/Mek signaling cascade. This is exemplified by the presence of the activated form of MAPK during involution in the TGFαTG and Stat5aKOTGFα mice. Persistent activation and phosphorylation of Stat5a in the TGFαTG mice could occur through three distinct mechanisms. The EGFR can directly phosphorylate Stat1 and Stat3 in the absence of Jak activation (46–48). Although direct phosphorylation of Stat5a by the EGFR cannot be ruled out, it was not possible to coimmunoprecipitate this receptor with anti-Stat5a antibodies in the TGFαTG mammary gland (data not shown). Secondly, stimulation of MAPK by EGF can lead to phosphorylation and nuclear translocation of the Stat5a isoform Stat5b in the liver (21). Support for this mechanism comes from results describing that Stat5a and its shorter isoforms can be tyrosine- and serine-phosphorylated by prolactin (18), and Stat5a and Stat5b can be tyrosine- and serine-phosphorylated by prolactin-independent, MAPK-dependent mechanisms (22, 49). A direct interaction was recently demonstrated between MAPK(ERK1/2) and Stat5a in growth hormone-dependent signaling (23). Growth hormone is known to influence mammary gland involution, although this effect is not as dominant as that observed for prolactin (2). Thirdly, EGFR could activate Stat5a through Jak-2. The EGFR is known to use Jak-1 to activate Stat1 and Stat3 (14, 50) and can modulate Stat5 expression in mammary epithelial cells (51). Theoretically, the constitutively active EGFR in the TGFαTG and Stat5aKOTGFα mice could stimulate Jak-2, although this was not formally proven. Alternatively, EGFR could stimulate a secondary Jak, which inappropriately uses Stat5a as a substrate that has been demonstrated in IFN-γ signaling (52, 53). Presumably, the stimulation of the EGFR kinase in the presence of the TGF-α transgene leads to phosphorylation and activation of a downstream kinase, possibly including a Jak, that maintains Stat5a in its phosphorylated state. We suggest that one or more of these mechanisms regulate the phosphorylation of Stat5a and control apoptosis in the mouse mammary gland.

Persistent Stat5a Activity Contributes to Mouse Mammary Tumorigenesis, and Its Absence Acts to Delay Hyperplasitic Epithelial Formation. One of the dominant pathways commonly altered in breast cancer is the EGF signal transduction pathway (25, 54). Gene deletion studies in mice have demonstrated that the EGFR is not dispensable for normal mammary development (55, 56), and that aberrant signaling through this receptor induces neoplastic growth and frank tumors (26, 57–59). Using an EGF-dependent transgenic mouse model, we can now show that the persistent activation of Stat5a is linked to the transformation of the mammary gland. We suggest that the transformation of the mammary epithelium occurs at the expense of a loss of cell survival regulation, which is contingent on the persistent phosphorylation of Stat5a and permits the inappropriate survival of epithelial cells. The appearance of tumors and hyperplasias in Stat5aKOTGFα mice suggests that the absence of Stat5a will not completely block mammary epithelial transformation, and that the TGF-α transgene can activate alternative (non-Stat5a) pathways to bypass PCD and involution and support tumor formation. Such pathways may include Stat5b, which, under specific circumstances, substitutes for Stat5a (60). The involvement of a single gland in a majority of the Stat5aKOTGFα tumors suggests that significant numbers of potentially transformed cells are destroyed during involution. This loss of cells probably contributes to the observed lower rate of tumor formation and the delay in initial tumor appearance. In spite of single gland involvement, survival of the Stat5aKOTGFα mice was no greater than that observed for the TGFαTG mice after initial tumor appearance.

Our findings that Stat5a is a survival factor and that its absence reduces mammary tumorigenesis support findings from other settings. Prolactin and other type 1 cytokines do possess mitogenic activity, implicating the associated Stat molecules with contributing to the mitogenesis of these organs (13). Constitutive Stat activity has been observed in cells transformed by oncogenic viruses (32) and oncogenes (61) and in leukemia cells (29). Moreover, constitutive activation of Jak expression can lead to transformation (27, 62).

Stat5a Is a Survival Factor for the Mammary Epithelium. The absence of Stat5a disrupts the prolactin-mediated survival of the mammary epithelium. Whereas overexpression of TGF-α in the presence of an intact Stat5a results in delayed involution, apoptosis and involution in the absence of Stat5a lead to elimination of the majority of the epithelium. The dephosphorylation or lack of *de novo* phosphorylation and inactivation of Stat5a that occur during involution in the WT gland are mimicked by the absence of Stat5a in the Stat5aKOTGFα mice. Therefore, we propose that the presence of the activated form of this transcription factor prolongs cell survival, and its inactivation or deletion permits PCD to occur. These experiments demonstrate that Stat5a can act as a survival factor for the epithelium of the mammary gland. In addition, they illustrate that the inhibition and/or disruption of the mechanisms that regulate this involution process are critical in mammary gland tumorigenesis.

The conclusions from this study lead us to hypothesize that the inhibition or disruption of Stat5a phosphorylation can lead to protection from transformation in the mammary gland. The application of Stat and/or Jak-specific tyrosine or serine kinase inhibitors may be an approach to blunt the development of mammary tumors originating from EGFR deregulation.

Materials and Methods

Materials. The TGF-α cDNA probe was a kind gift from Dr. David Lee (Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC). The generation of the Stat5a antibodies has been described previously (63). Phosphorylated MAPK antibody was purchased from Promega (Madison, WI). Anti-phosphotyrosine antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Goat antirabbit and rabbit antimouse secondary antibodies were purchased from Transduction Laboratories (Lexington, KY).

Generation of TGFαTG and Stat5aKOTGFα Mice. WAP-TGFαTG mice were a gift of Dr. Eric Sandgren (University of Wisconsin, Madison, WI Ref. 26). The generation of the *Stat5a*-null mice has been described previously (4). TGFαTG mice were interbred with *Stat5a*-null mice (SvEv129/C57B6) to generate F_1 founders that were hemizygous for *Stat5a* deletion (Stat5aHT) and transgenic for TGF-α expression. Female Stat5aHTTGFα mice were bred with Stat5aHTTGFα males to generate offspring that were transgenic for TGF-α and homozygous for deletion of the *Stat5a* gene. These mice were backcrossed for five generations to generate a pure inbred Stat5aKOTGFα strain of mice. The same generation TGFαTG and control WT littermates was used for molecular analyses. Confirmation of the presence of the TGF-α transgene was performed by PCR analysis with TGF-α forward primer 5′-TGTCAGGCTCTGGAGAA-CAGC-3′ and reverse primer 5′-CACAGCGAACACCCACGTACC-3′. *Stat5a* PCR was performed with two sets of primers to knockout (null) and WT alleles: (*a*) *Stat5a* forward (5′-CTGGATTGACGTTTCTTACCTG-3′) and *Stat5a* reverse (5′-TGGAGTCAACTAGTCTGTCTCT-3′) and (*b*) Neo forward (5′-AGAGGCTATTCGGCTATGACTG-3′) and *Neo* reverse 5′-TTCGTCCAGATCATCCTGATC-3′. PCR for all primers was performed with a denaturing step of 94°C for 3 min, followed by 30 cycles of 94°C for 40 s, 56°C for 40 s, and 68°C for 40 s, followed by 10 min at 68°C. Genotype of the mice was confirmed after tissue collection by Northern and Western blot analysis for TGF-α gene expression and absence of Stat5a protein expression, respectively. All animals were housed and handled according to the approved protocol established by the Institutional Animal Care and Use Committee and NIH guidelines.

Mammary Gland Collection. Mammary glands were surgically removed from anesthetized and cervically dislocated mice at day 18 of pregnancy and days 1, 3, and 7 of involution. Day 1 of involution was designated as 24 h after the morning that the pups were born. Pups were immediately removed from the dam after birth and fostered onto a WT mother. The mammary lymph node was removed before homogenization of all glands. Tissues were prepared immediately for RNA and protein extraction, as described previously (42). Whole (number 4) inguinal or (number 3) thoracic mammary glands were surgically excised from mice, spread on Omniset tissue cages (Fisher Scientific, Pittsburgh, PA), fixed for 5 h in Tellyzinckys fixative, and stored in 70% ethanol until processed by standard embedding and sectioning techniques onto Probe-On Plus slides (Fisher Scientific). Sections were stained with H&E.

Immunoprecipitations and Western Blot Analysis. Preparation of protein extracts and immunoprecipitations have been described previously (42). Briefly, 2 mg of fresh and frozen tissue were homogenized in 2 ml of lysis buffer with protease inhibitors; phenylmethylsulfonyl fluoride, leupeptin, and aprotinin at 50 μg/ml on ice. Protein lysates were rocked for 1 h at 4°C and then cleared by centrifugation at 14,000 $\times$ *g* for 15 min. The supernatants were removed, mixed with 2$\times$ loading buffer, and heated to 90°C for 3 min. Samples were spun briefly, electrophoresed under denaturing conditions on 8% precast tris-glycine gels, and transferred to polyvinylidene difluoride membranes according to manufacturer's protocol (Novex, San Diego, CA). Western blot analysis was performed essentially as described with the following exceptions; primary antibody (Stat5a, 1:20,000 dilution; Stat5b, 1:10,000 dilution; anti-phosphotyrosine, 1:5,000 dilution; anti-MAPK, 1:5,000 dilution) was incubated overnight at 4°C with gentle rocking, and all incubations with antibodies and initial blocking were performed with 3% nonfat dried milk in 1$\times$ TBST. Detection was performed with the enhanced chemiluminescence kit according to manufacturer's protocol (Amersham) and exposed to Kodak (Rochester, NY) MR autoradiography film. Exposure times are between 1 s and 2 min. Immunoprecipitations with anti-Stat5a antibodies were carried out as described previously (4). Stripping was performed by incubating blots at 56°C in 6.25 mM Tris-HCl (pH 6.8), 2% SDS, and 1% β-mercaptoethanol for 30 min. Blots were washed extensively in TBST and then blocked with 3% nonfat dried milk in TBST.

Northern Blot Analysis. Total RNA was isolated from fresh tissue by homogenization in lysis buffer as described previously (63). RNA was quantified by spectrophotometry and prepared for Northern blot analysis by heating in loading buffer at 65°C. Total RNA was separated by 1.5% agarose gel electrophoresis and transferred to Hybond N+ (Amersham) nylon membrane by capillary transfer with 10 $\times$ SSC. After overnight transfer, the membrane was UV-irradiated and hybridized to each cDNA probe in Quikhyb (Stratagene, La Jolla, CA). Hybridization for the TGF-α cDNA probe was performed in Quikhyb (Stratagene) at 65°C for 16 h, followed by two washes in 1 $\times$ SSC and 0.5% SDS for 30 min, followed by one wash in 0.1 $\times$ SSC and 0.5% SDS for 30 min at 56°C. Blots were hybridized for 16 h and then washed twice in 1 $\times$ SSC and 0.5% SDS for 30 min, followed by one wash in 0.1 $\times$ SSC and 0.5% SDS for 30 min. cDNA and oligo probes were random-primed and Klenow-labeled with [α-^{32}P]dCTP. After hybridization and washing, blots were exposed from 30 min to 24 h, as specifically described in the figure legends, to Kodak MR autoradiography film at $-$70°C.

BrdUrd and TUNEL Assays. Protocols for BrdUrd and TUNEL analysis have been described elsewhere (64). Mice were injected 2 h before sacrifice with 20 μg/g body weight BrdUrd labeling reagent, as described by the manufacturer (Amersham). Each proliferation and apoptosis sample counted represents a minimum of three random fields (at $\times$200) and a minimum of 1000 total cells/section for each mouse. A minimum of three mice per timepoint were collected and analyzed. The total number of cells counted for proliferation assay is 6.5 $\times$ 10^5. The total number of cells counted for the apoptosis assay is 7.5 $\times$ 10^5. The number of mice collected and the number of mice analyzed are listed by genotype and by timepoints (pregnancy day 18/involution day 1/involution day 3/involution day 7) for apoptosis and proliferation, respectively: KOTG, 4/4/4/3 and 4/3/3/3; TG, 3/5/3/5 and 3/3/3/5; WT, 3/4/5/3/ and 3/3/3/3; NTGKO, 3/3/2/3 and 3/3/3/3.

Hyperplasia and Tumor Analysis. Mice were palpated at the 18th day of pregnancy for mammary hyperplasia, hypertrophy, and tumors. Animals were scored for the presence of either hyperplasia or tumor at every pregnancy. Most animals were sacrificed at the third pregnancy by anesthesia followed by cervical dislocation. In studies that required long-term breeding for tumor development, dams were rebred within 2 days after birth and removal of the pups.

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Abstract to poster presentation at the Breast Cancer think Tank Symposium Held At Chantilly Virginia

Signal Transducer and Activator of Transcription 5a (Stat5a) Regulates Tumorigenesis and Epithelial Cell Survival in the Mouse Mammary Gland

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The mammary gland undergoes extensive tissue remodeling and cell death at the end of lactation in a process known as involution. We present evidence that the prolactin-activated transcription factor, Stat5a has a crucial role in the regulation of cell death during mammary gland involution and can affect the progression of epidermal growth factor (EGF)-dependent mammary tumorigenesis. In a transforming growth factor-alpha (TGFα) transgenic mouse model, that exhibited delayed mammary gland involution, the absence of Stat5a facilitated involution-associated changes in morphology of the gland and the extent and timing of programmed cell death (PCD). These Stat5a-dependent changes also altered EGF receptor-initiated mammary gland tumorigenesis. Overexpression of the TGFα transgene in the mammary epithelium reproducibly generated mammary hyperplasia and tumors. In the presence of TGFα overexpression, complete genetic deletion of Stat5a reduced the rate of tumor formation and delayed initial mammary tumor development by 6 weeks. These observations demonstrate that Stat5a is a survival factor and its presence is required for the epithelium of the mammary gland to resist regression and involution-mediated apoptosis. We suggest that Stat5a is one of the antecedent, locally-acting, molecules that initiate the process of epithelial regression and reorganization during involution. In addition, these studies suggest that disruption of the pathway which leads to Stat5a activation can affect the progression of tumorigenesis in the mammary gland.
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Stat5a Influences Mammary Epithelial Cell Survival and Tumorigenesis

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The *in vivo* relationship between the epidermal growth factor (EGF) and prolactin/Jak/Stat signaling pathways in mammary gland development and tumorigenesis was explored in transgenic mice which overexpress the TGF alpha gene (TGFαTG) in the mammary epithelium and lack the prolactin-activated transcription factor, Stat5a (Stat5aKO). We present evidence that Stat5a has a crucial role in the regulation of cell death during mammary gland involution and the development of EGF-dependent tumorigenesis. TGFαTG mice exhibit delayed mammary gland involution and the absence of Stat5a in the Stat5aKO/TGFαTG gland facilitated involution-associated changes in morphology and the extent and timing of programmed cell death in the mammary gland. Stat5a remained phosphorylated through involution in the TGFαTG mammary glands suggesting that Stat5a is constituitively phosphorylated and "activated" by the EGF signaling pathway. These results suggested that the TGFα epithelium is prevented from progressing into the normal pattern of involution and apoptosis by the "activated" form of Stat5a. Alternatively, the presence of activated Stat5a is required for the epithelium of the TGFαTG mammary gland to resist regression and involution-mediated apoptosis.

These Stat5a-dependent changes also affected EGF-receptor initiated (EGFR) mammary gland tumorigenesis. Overexpression of the TGFα transgene in the mammary epithelium reproducibly generates mammary hyperplasia and tumors. In the presence of the activated EGFR, deletion of Stat5a delayed initial hyperplasia and mammary tumor development by 6 weeks. These observations demonstrate that Stat5a is a survival factor and its presence is required for the epithelium of the mammary gland to resist regression and involution-mediated apoptosis. This effect on involution can influence the development of mammary gland tumorigenesis. We are currently utilizing specific kinase inhibitors to block the phosphorylation of Stat5a by the EGFR signaling pathway in order to determine the mechanism of Stat5a activation. This work is supported by a grant from the DoD, USMRMC to R.C.H. [DAMD17-99-1-9328]

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Transforming growth factor alpha and mouse models of human breast cancer

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Transforming growth factor alpha (TGFα) is a principal molecule in the normal and neoplastic development of the mammary gland. Binding of TGFα to the epidermal growth factor receptor (EGFR), activates the EGFRs' endogenous tyrosine kinase activity and stimulates growth of the epithelium in the virgin and pregnant mouse mammary gland. TGFα expression can be detected in breast cancer cells *in vivo* and *in vitro* and overexpression can elicit partial transformation or immortalized human and rodent mammary epithelial cells. Despite evidence implicating TGFα in the development of mammary neoplasia, the actual mechanism of TGFα-induced transformation is unclear. Transgenic mouse models targeting heterologus TGFα to the mammary gland have established TGFα overexpression can induce hyperproliferation, hyperplasia and occasional carcinoma. These transgenic studies demonstrated a facilitating, proliferative role for TGFα in the development of neoplasia and implicated several oncogenes that can cooperate with TGFα to transform the mammary epithelium. From studies of EGFR signaling pathways, inhibitory and modulating agents such as anti-EGFR antibodies and specific kinases inhibitors have been used to block the action of this pathway and prevent the development of TGFα-induced neoplasia and tumor formation. Studies in Stat5a knockout mice have established that the JAK2/Stat5a pathway can facilitate the survival of the mammary epithelium and can impact the progression of TGFα-mandated mammary tumorigenesis. Together these experiments indicate that TGFα and the EGFR signaling pathway are potentially amenable to therapies for treatment of human breast disease. *Oncogene* (2000) **19,** 1085–1091.

Keywords: TGF alpha; mammary gland; transformation; cancer; mouse models; transgenic

Introduction

Development and progression of breast cancer, like many other types of human cancer, is dependent on the progressive corruption and alteration of normal signaling pathways. Signaling mechanisms that transmit growth signals are a predominant target for carcinogenic and oncogenic alterations as they often confer a growth advantage to the pre-neoplastic cell. One of the primary growth factor receptors involved in normal and neoplastic development of the mammary gland is the EGFR and its extended family of related receptors and peptide ligands (Table 1). EGFR and the ligand TGFα, have expression levels altered in the development of cancer in numerous issues. The related family of ligands that bind to these receptors; EGF, heregulin, amphiregulin, and cripto also display alterations in their expression and activity within the pre-neoplastic and transformed cell (for a review see (Dickson and Lippman, 1995; Gullick *et al.*, 1999; Schroeder and Lee, 1997)). Despite the well characterized role of the EGFR and TGFα in normal mammary growth signaling, in transformation of mammary epithelial cells *in vitro* and *in vivo* and their elevated expression in some breast cancers, the specific alterations of the EGFR signaling mechanism that lead to TGFα-initiated neoplasia are poorly understood. In an attempt to elucidate these alterations, several transgenic mouse models have been generated to explore the role of TGFα and EGFR in mammary neoplasia. This review will focus on TGFα and its role in transformation of the mammary gland. In addition, it will examine those studies that have attempted to identify the mechanisms of TGFα action in the mammary gland and those molecules that are capable of cooperatively interacting with TGFα to promote tumorigenesis.

TGFα and EGFR expression coincides with normal proliferation in vivo

TGFα is structurally and functionally similar to EGF; the peptides share a 42% identity and can elicit the same biological effects in cultured mammary epithelial cells and explants (Daniel and Silberstein, 1985; Salomon *et al.*, 1987; Vonderhaar, 1987). TGFα is often co-expressed with the EGFR and binding to the receptor activates the EGFRs' endogenous tyrosine kinase activity. Mammary epithelial cell lines can be stimulated to proliferate by TGFα (Smith *et al.*, 1989; Zajchowski and Sager, 1991) and it can act as an autocrine growth factor in normal and immortalized human mammary epithelial cells. TGFα autocrine and proliferative activity can be blocked in these cells by an anti-EGFR antibody (Bates *et al.*, 1990; Kenney *et al.*, 1993).

In normal mammary gland development, TGFα and EGF transcripts can be detected in the ductal and lobuloalveolar stages. The virgin mouse mammary gland expresses TGFα in the proliferative cap cells and the stromal fibroblasts around the neck of the terminal endbud, whereas EGF expression is localized

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Table 1

Ligand	*Receptor*
EGF-Like	
EGF	EGFR
TGFα	EGFR
Amphiregulin	EGFR
Epiregulin	EGFR ErbB-4
Betacellulin	EGFR ErbB-3 ErbB-4
Heparin binding-EGF	EGFR ErbB-3 ErbB-4
Heregulin/neuregulins	
Heregulin-1 (α and β)	ErbB-3 ErbB-4
Heregulin-2 (α and β)	ErbB-3 ErbB-4
Heregulin-3	?
Heregulin-4	ErbB-4
Glial growth factor	?
Cripto	
Cripto-1	? unique receptor and erbB4
Cripto-3	?

to the luminal ductal epithelium (Snedeker *et al.*, 1991). In the absence of ovarian steroids, exogenous TGFα and EGF can stimulate ductal growth of the mouse mammary epithelium suggesting that they can act independently of secondary signals (Snedeker *et al.*, 1991). EGF and TGFα mRNA is present in pregnant and lactating rat and human mammary glands and increases 2–3-fold over virgin levels at pregnancy (Liscia *et al.*, 1990). The EGFR is required for ductal development (Wiesen *et al.*, 1999) and can be detected in both the stromal and epithelial components of the virgin mammary gland (Coleman *et al.*, 1988). In the pregnant gland, a rapid increase is observed during midpregnancy precisely at the time of extensive cellular proliferation (Edery *et al.*, 1985). After pregnancy, the levels of EGFR decrease significantly. The coincident expression of TGFα and its receptor with the proliferative phases of mammary epithelial growth and TGFαs' direct mitogenic effect on epithelium *in vitro* and *in vivo* confirms the functional role for this signaling dyad in the development and proliferation of the mammary epithelium.

TGFα expression is associated with human breast cancer

TGFα is expressed and mitotically active in numerous breast cancer cell lines and has been directly implicated as a modulator of transformation *in vivo* (Borellini and Oka, 1989; Daniel and Silberstein, 1985; de Jong *et al.*, 1998a; Salomon *et al.*, 1984; Valverius *et al.*, 1989). In fact, the discovery of TGFα was based on its ability to transform retrovirally-infected cultured fibroblasts (Todaro *et al.*, 1980; de Larco and Todaro, 1978). Expression of TGFα has been identified in pleural effusions from normal mammary gland (Arteaga *et al.*, 1988), in invasive ductal carcinoma (Pilichowska *et al.*, 1997) and correlates with increased neo-angiogensis (de Jong *et al.*, 1998b) in breast tumors. Carcinomas of the breast that have higher level expression of TGFα also express high levels of EGFR, implicating a functional role for the TGFα/EGFR autocrine loop in tumors (Umekita *et al.*, 1992). The correlation between levels of EGFR expression and neoplastic transformation *in vivo* is controversial (Gullick and Srinivasan, 1998). Robertson and colleagues demonstrated that 40–60% of neoplasias examined in one study displayed normal levels of EGFR expression (Robertson *et al.*, 1996). Only a small proportion of breast cancers has elevated levels of EGFR (Slamon *et al.*, 1987), or amplification of EGFR (Peters and Wolff, 1983). This data implies that alterations in EGFR ligands may be more influential on the activity of the EGFR than changes in the levels of receptor expression itself. Alternatively, the formation of heterodimers between EGFR and the other erbB family members may play a role in effecting differential activities in tumorigenesis of the breast.

TGFα can modulate cellular transformation in breast cancer cell lines. In the immortalized human breast cancer cell line, MCF-7, TGFα transfected cells that expressed EGFR were stimulated to grow in a colony forming assay (Ciardiello *et al.*, 1990). In a parallel study with fully transformed MCF-7 cells that lacked EGFR, TGFα transfection did not effect a positive growth advantage (Clarke *et al.*, 1989). Overexpression of TGFα in the immortalized mouse mammary cell line, NOG-8, generated anchorage independent growth but colonies failed to form tumors in nude mice (Shankar *et al.*, 1989). Therefore, expression of TGFα is associated with the ability to stimulate growth, while overexpression is allied with partial transformation *in vitro* and correlated with appearance of carcinomas *in vivo*.

TGFα transgenic mouse models of breast cancer

Mouse models have been developed to study the role of TGFα in the transformation and development of the mammary gland. Several promoters have been utilized to target TGFα both non-specifically and specifically to the mammary gland. These mouse models have established the importance of TGFα in the early stages of neoplastic development of the mammary gland.

Initial studies demonstrating the neoplasia-promoting activity of TGFα were described in mice expressing human TGFα under the control of the zinc-inducible metallothionein (MT) promoter (Jhappan *et al.*, 1990). Despite the fact that expression of the transgene was low in the mammary gland and was generally not inducible, these mice displayed increased cellular proliferation and delayed epithelial penetration of the stromal fatpad during ductal development. In a second parallel study, using the MT promoter directing the expression of rat TGFα, Sandgren and colleagues noticed that mice that had passed through multiple pregnancies developed hyperplastic nodules and dysplasia of the mammary epithelium (Sandgren *et al.*, 1990). Only one mouse developed a secretory adenocarcinoma. These studies demonstrated that there was an association between the *in vivo* expression of TGFα and the development of mammary hyperplasia.

A study directing TGFα expression specifically to the mammary gland with the mouse mammary tumor virus (MMTV) promoter demonstrated unequivocally the growth and neoplasia promoting activity of TGFα (Matsui *et al.*, 1990). Precocious alveolar development, hyperproliferation and hyperplasias were apparent in the mature virgin mouse. The alveolar hyperplasia was present in glands from mature virgin mice, but not in

immature virgin mice, suggesting a requirement for secondary inputs from systemic hormones for any TGFα-dependent hyperplasia to occur. Hyperplastic alveolar nodules and cysts were prominent in the multiparous animal and increased in hyperplastic and dysplastic character with the number of pregnancies. One multiparous mouse developed adenocarcinomas although none were metastatic. A second study was performed with the MMTV-TGFα mice that studied in greater detail the generation of hyperplasia and tumors (Halter *et al.*, 1992). At one year, 65% of multiparous and 45% of virgin mice displayed hyperplasia. By 16 months, 40% of the multiparous and 30% of the virgin mice and mice had generated tumors. These studies demonstrated that TGFα could stimulate the proliferation of the mammary epithelium and generate proliferative late pregnancy mammary gland phenotypes in early pregnant animals. In addition, the hyperplasia, dysplasia and frank carcinoma observed with this mouse model suggested that there was a multistage nature to the progression of TGFα-initiated transformation.

A more dramatic mammary phenotype was achieved by targeting rat TGFα exclusively to the mammary gland with the WAP-TGFα transgenic mouse (Sandgren *et al.*, 1995). This transgenic model achieved high level TGFα expression during pregnancy and lactation and displayed similar proliferative mammary gland phenotypes as described in the MT and MMTV transgenic models but with an increased incidence and decreased latency of tumors. In addition, the process of involution, in which the gland absorbs and reorganizes a significant portion of its epithelial structure, was delayed in these mice as it was in the original MT mice (Sandgren *et al.*, 1990). Latency of tumor appearance was decreased in comparison to the MMTV-TGFα model, but still required several rounds of pregnancy for initial tumor development. A majority of the tumors were well-differentiated glandular adenomas or carcinomas (53%) and fibroadenomas (35%) and retained the glandular characteristics of late pregnant mammary epithelium. The appearance of multiple types of tumor and the retention of normal glandular morphologies suggested that these different types were stages of TGFα-dependent tumor progression. Interestingly, the levels of cyclinD1 were elevated in the WAP-TGFα transgenic glands. The targeted overexpression of this cell cycle regulatory molecule is known to effect mammary transformation (Wang *et al.*, 1994) and is in a chromosomal region frequently rearranged in human breast cancer (Dickson *et al.*, 1995; Gillett *et al.*, 1994, 1996, 1999). This indicates that cyclinD1 may be a cooperative factor in the development of TGFα induced mammary carcinogenesis. The delay in involution was suggested as a mechanism for promoting transformation of the mammary gland as it provided an expanded population of proliferative epithelial cells that could be predisposed to transformation. This seminal paper demonstrated TGFα could transform the mammary

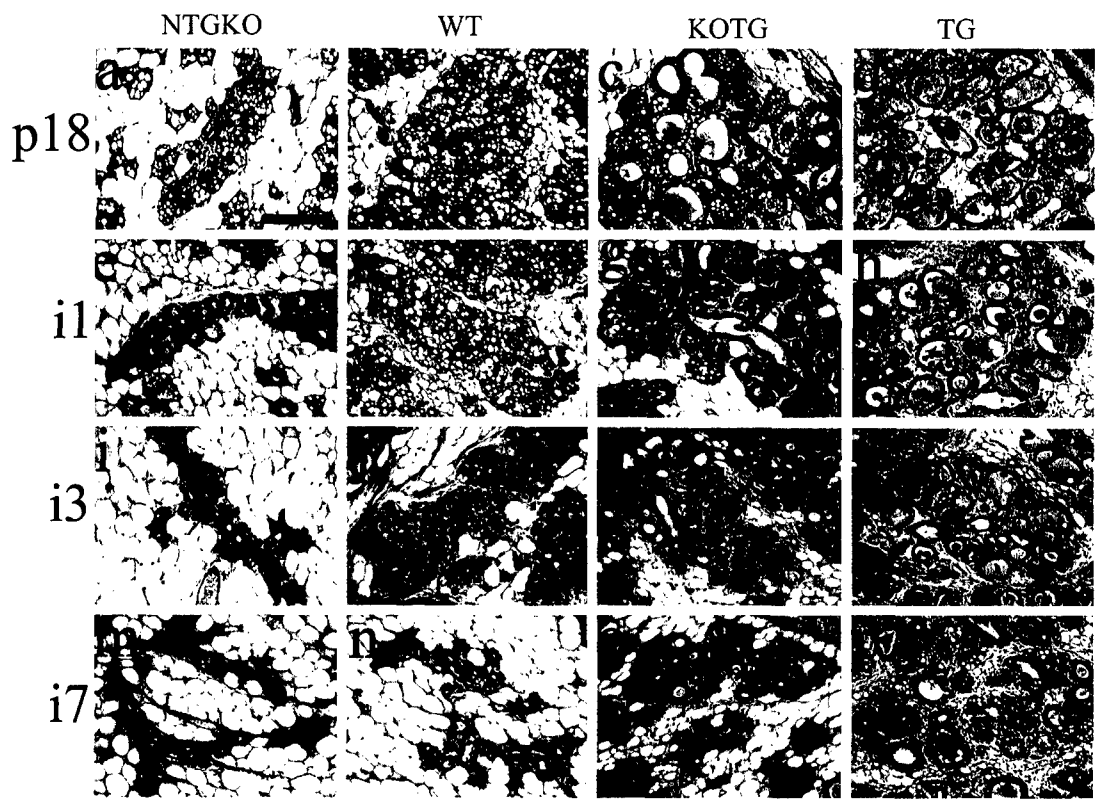

Figure 1 Involution is enhanced in the absence of Stat5a. Hemotoxylin and eosin staining of inguinal mammary glands from Stat5a null non-transgenic (NTGKO), wildtype (WT), Stat5a null TGFα transgenic (KOTG) and TGFα transgenic (TG) mice at 18 days of pregnancy (p18/a–d), days 1 (i1/e–h), 3 (i3/i–l) and 7 (i7/m–p) of involution. Samples were collected from mice in or immediately after their first pregnancy. Compare epithelial condensation at day 3 and 7 of involution in the wildtype (1-j) versus the KOTG (1-k) and TG (1-l). Magnification is defined by the bar in a = 200 μm. Reproduced with permission (Humphreys and Hennighausen, 1999)

epithelium and suggested a possible mechanism for neoplastic development. The observation that TGFα could cause a delay in involution was supported by results from a separate study with MT-TGFα mice (Smith *et al.*, 1995). An increase in DNA synthesis in lactation was accompanied by a significant decrease in apoptotic cells after 2 days of involution. Involution was also affected in a mouse model utilizing WAP-TGFα and the Stat5a knockout mouse (Humphreys and Hennighausen, 1999). In the absence of Stat5a, apoptosis levels rose in the mammary gland during pregnancy and involution. This increase in apoptosis enhanced involution of the WAP-TGFα gland (Figure 1) and increased the latency of WAP-TGFα-induced tumors. This result demonstrated the WAP-TGFα-induced delay in involution and tumor formation, could be abrogated by a downstream signaling molecule that regulates cell death in the mammary epithelium.

Subsequent studies examined the role that oncogenes may play in acting cooperatively with TGFα to promote mammary transformation. The oncogene c-*myc* is overexpressed in 25–30% of breast cancer (Bonilla *et al.*, 1988; Callahan and Campbell, 1989; Mariani-Costantini *et al.*, 1988, 1989; Morse *et al.*, 1988) and in rodent mammary epithelial cell lines transfected with myc, TGFα could cooperate to support a transformed phenotype (Telang *et al.*, 1990). Mice that overexpressed both c-*myc* and TGFα in the mammary gland had an increased tumor incidence and decreased tumor latency when compared to c-*myc* transgenics (Amundadottir *et al.*, 1995). A second paper utilizing the same bitransgenic mice demonstrated that the synergism between these two proteins was due to a cooperative growth stimulus and inhibition of c-*myc*-induced apoptosis by TGFα (Amundadottir *et al.*, 1996). Apoptotic tumors were present exclusively in mice that expressed only c-*myc*. In cell lines derived from these tumors, exogenous TGFα could inhibit apoptosis. These data again implicated TGFα as a survival factor in the mammary epithelium. Interestingly, tumor cells from the mammary glands of these bitransgenic mice could only become apoptotic when exposed to a specific inhibitor of the EGFR kinase pathway. This result suggested that an intact TGFα/EGFR autocrine loop was required to mediate the survival effects of TGFα.

Interaction between TGFα and the proto-oncogene *neu* was examined in bitransgenic mice expressing both

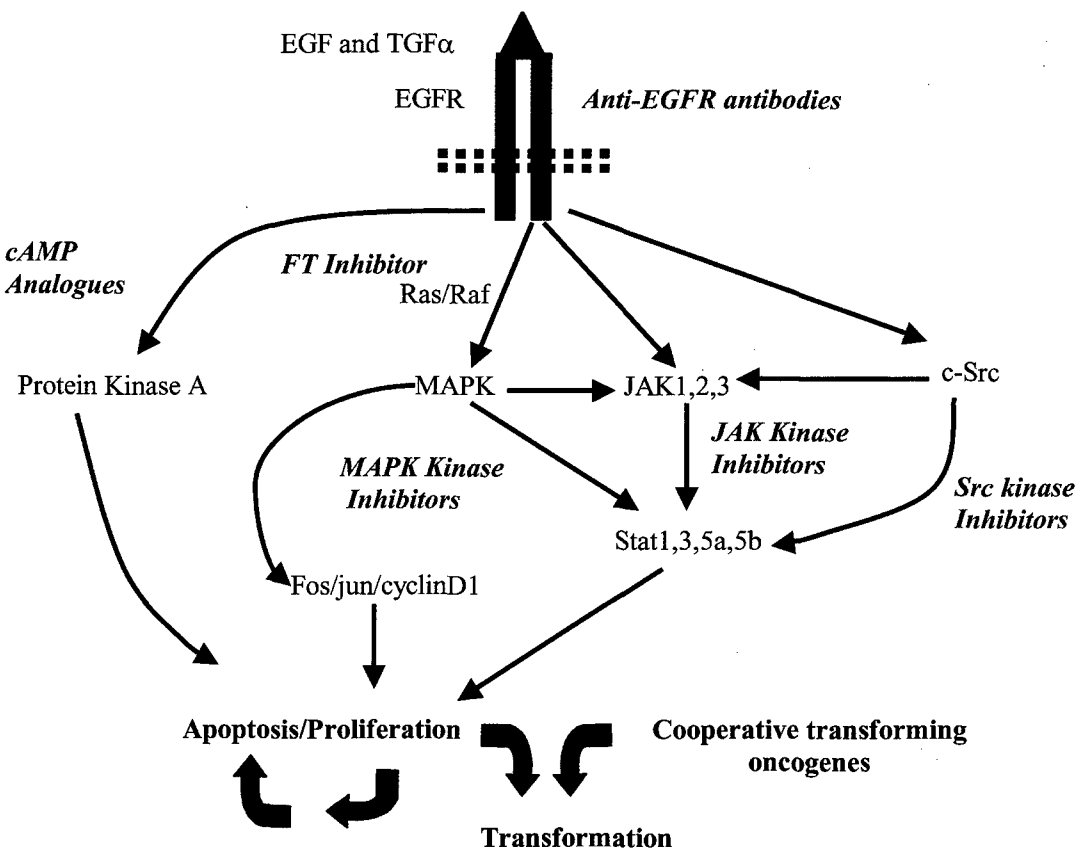

Figure 2 Model of the TGFα/EGF signal transduction pathway and EGFR signaling inhibitors that affect EGFR-mediated transformation. EGF and TGFα binding to the EGFR stimulate the activation of the endogenous receptor tyrosine kinase. The membrane-bound EGFR kinase activates one of several intracellular signal transduction pathways including the protein kinase A, Ras/Raf/MAPK, c-*src* and Jak/Stat pathways. Direct or indirect phosphorylation and activation of one or more of the Stat proteins, 1, 3, 5a, and 5b can be achieved through several of these mechanisms. The activating kinase, attributes of the targeted Stat and phosphorylated residue can elicit distinct functional consequences for the cell. Multiple intercellular pathways can lead to activation of the Stats and other nuclear factors like myc and cyclinD1. Each of these pathways can be blocked by specific inhibitors (shown in red Italics). The proliferative stimulus provided by TGFα and EGF in cooperation with transforming oncogenes can lead to cellular transformation. Consequently, a clear understanding of the mechanisms involved and the use of multifocal inhibitors of signaling intermediaries is critical for effectual inhibition of EGFR signal transduction. Some alternative and intermediary intercellular signaling molecules have been omitted for clarity

genes under the control of the MMTV promoter (Muller *et al.*, 1996). The *neu* proto-oncogene is a member of the EGFR family and although it does not bind to TGFα or EGF, it can form heterodimers with the EGFR and it is often found overexpressed and amplified in human breast cancers (Slamon *et al.*, 1987). Tumor latency was decreased in the bitransgenic mice, compared to the TGFα and *neu*, monotransgenic lines. At 150 days 95% of the bitransgenic mice had mammary tumors vs 6% and 35% for the TGFα and *neu* mice, respectively. Bitransgenic tumors were multifocal whereas mono-transgenic lines generated focal tumors and contained activated *neu*. This paper suggested a novel mechanism for TGFα cooperativity involving the transactivation of *neu* through the EGFR. Treatment of MMTV-TGFα mice with the tumor promoter 7, 12 dimethyl benzanthracene demonstrated that TGFα could accelerate tumor formation and the authors suggested that TGFα could act as a tumor promoter (Coffey *et al.*, 1994).

These studies on the mechanism of TGFα-mediated tumorigenesis and cooperativity in the mammary gland demonstrate that the overexpression of TGFα gives the preneoplastic mammary epithelium a proliferative advantage but in and of itself is not a transforming event. Secondary events, like the activation of proto-oncogenes, can significantly increase the efficiency with which TGFα can transform the mammary epithelium. Importantly, these studies support the theory that TGFα's influence on the regulation of apoptosis in the mammary gland is a possible mechanism of promoting survival of the neoplastic cell.

Disruption of TGFα signaling and potential therapies for breast cancer

The EGFR is required for mammary gland ductal development (Wiesen *et al.*, 1999; Xie *et al.*, 1997) and, as described previously, is aberrantly expressed in 40% of human breast cancers and occasionally overexpressed in those tumors with poor prognosis. This receptor can interact with several distinct ligands (Table 1) and activates different intracellular and nuclear signaling pathways (Figure 2). Substrates for this type 1 receptor tyrosine kinase are numerous and demonstrate the influence of EGFR on the regulation of cellular growth. Importantly, it is clear that this receptor plays a central role in the development and progression of human breast cancer. Therefore, insight into EGFR signal transduction and the mechanism of substrate selection and activation are critical to understanding the development of breast cancer. The EGFR and its interaction with TGFα has been and continues to be a target for potential anti-cancer therapies that aim to regulate its activity and signaling mechanisms.

A recent study demonstrated that inhibition of the signal from EGFR to its intracellular signaling molecules is critical in blocking tumor progression. Treatment of MMTV-TGFα transgenic mice with a farnesyl transferase inhibitor, which blocks Ras function, demonstrated a significant regression of mammary tumors (Norgaard *et al.*, 1999). This inhibitory effect was ineffectual after tumor acceleration from multipregnancy or after treatment with the carcinogen DMBA supporting the theory that that EGFR signaling is important in the initial stage of neoplastic development. The EGFR can activate the intracellular tyrosine kinases Jak1 and the transcription factors Stat1, 3 and 5b in response to growth signaling from EGF (Leonard and O'Shea, 1998). Recent data has demonstrated that growth hormone activated Ras/Raf/MAPK pathway can directly activate the normally cytokine-activated pathway of Stat5a (Pircher *et al.*, 1999) and EGF can phosphorylate Stat5a through c-*src* (Olayioye *et al.*, 1999). Stat5a, a prolactin-activated transcription factor, has an established role in the development and differentiation of the lactation-competent mammary gland (Liu *et al.*, 1987) but is also associated with signaling in tumor cells (Hayakawa *et al.*, 1998; Richer *et al.*, 1998; Yu *et al.*, 1997; Zhang *et al.*, 1996). Stat5a-null mice interbred with WAP-TGFα transgenic mice revealed inhibition of TGFα-dependent MAPK activation and TGFα-dependent apoptosis inhibition after deletion of Stat5a. The increase in apoptosis permitted a more complete epithelial regression to occur at involution (Figure 1). A more complete regression deleted a significant number of potentially neoplastic cells and this impacted WAP-TGFα driven tumorigenesis. Stat5a null/WAP TGFα transgenic mice had an increase in tumor latency when compared to the WAP-TGFα transgenic. These mice displayed an increase in apoptosis before and during involution. These data suggested Stat5a was acting as a survival factor for the mammary epithelium by blocking the onset of apoptosis. Theoretically, the absence of Stat5a permitted apoptosis to occur and thereby diminished the pool of potential neoplastic cells that could become transformed (Humphreys and Hennighausen, 1999).

A novel mechanism of inhibition of TGFα activity was demonstrated recently in human mammary epithelial cells with an anti-metalloproteinase. Metalloproteinases are extracellular enzymes that cleave components of the extracellular matrix including the EGFR ligands; TGFα and EGF. This cleavage event releases them from the cell surface rendering the growth factor into an active form. These metalloproteinase inhibitors prevented TGFα release from the cell surface, blocked cell migration and decreased proliferation. Additionally, the metalloproteinase inhibitors reduced the growth of EGF-dependent tumor cell lines and could synergize with anti-EGFR antibodies (Dong *et al.*, 1999).

Therapies for inhibiting the action of EGFR and indirectly the action of TGFα, to block tumorigenesis are being explored. Current research into the action of molecules that may have a role in regulating the TGFα signaling pathway like EGFR-specific monoclonal antibodies (Ciardiello *et al.*, 1999), protein kinase A inhibitors, a combination of antibodies and chemotheraputic agents (Bianco *et al.*, 1997; Ciardiello *et al.*, 1996; Ciardiello and Tortora, 1998), antiestrogens like taxol, raloxifen, alone or in combination with antibodies like the humanized monoclonal antibody to *neu*; Herceptin (Brenner and Adams, 1999; Hanna *et al.*, 1999; Robertson, 1998; Ross and Fletcher, 1998), provide some promising results for future treatment of breast cancer.

Conclusions

Transgenic mouse model studies have given us insight into possible mechanism of TGFα-initiated breast cancer. Overexpression of TGFα may be an early prognostic factor in the initiation of this disease and in cooperation with secondary transforming events can lead to carcinoma. Importantly, the role of TGFα in the inhibition of apoptosis during normal development could lead to a critical understanding of the role this growth factor plays in normal and neoplastic development in the mammary gland. This growth factor pathway appears to be able to promote growth in the initial phase of neoplastic transformation although it lacks the dominating transformation ability observed with some of the other EGFR-related mouse models of mammary gland disease like MMTV/erb-2 (Guy *et al.*, 1992; Siegel *et al.*, 1999). In combination with an understanding of TGFα signaling mechanisms, inhibitory agents, which act on the receptor-ligand interaction or on the intracellular kinases stimulated by the EGFR, could lead to practical and efficacious treatment regimens for this disease.

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